

**NOVEL APPROACHES TO BREAST CANCER THERAPY:
EVALUATION OF NEXT GENERATION DENDRIMERS FOR
DRUG DELIVERY**

A Senior Scholars Thesis

by

MERIDETH SNOW

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2010

Major: Nutritional Science

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Research Advisor:

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Clinton Allred

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ABSTRACT

Novel Approaches to Breast Cancer Therapy: Evaluation of Next Generation Dendrimers for Drug Delivery. (April 2010)

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Breast cancer is the second leading cause of cancer deaths in women with approximately 40,000 patient deaths each year. Theranostics is an emerging area of cancer research in which chemically synthesized molecules are used to simultaneously deliver a diagnostic factor (e.g. DNA) and chemotherapeutic drug. This can be accomplished through the use of a dendrimer-based vector system that is capable of delivering DNA and a chemotherapeutic agent to the cancer cells. A previous study from our laboratory has shown that treating the cancer cells with a novel dendrimer construct (G2-NH₂) alone, without drug or DNA present, can be cytotoxic. The purpose of this study was to test the next generation of dendrimers for the ability to deliver DNA into cancer cells and their efficacy to induce cell death. MCF-7 cells were transfected with green fluorescent protein (GFP) plasmid DNA using dried or liquid forms of the second generation dendrimers (F2-1 and G2-5). Following transfection, fluorescence was measured to assess the transfection capability of the dendrimers. Dried forms of the dendrimers showed very little fluorescence while liquid forms of both dendrimers revealed low-level fluorescence. The transfection experiment was repeated for an additional set of

dendrimers (M6-24, M8-96, F4-2, F4-1, and F2-1). Dendrimer F4-2 showed the highest transfection efficiency. Additionally, cytotoxicity of the second generation dendrimers was measured. Dendrimers M8-96, M6-24, and F4-2 were cytotoxic at high concentrations while dendrimers F4-1 and F2-1 were not. The dendrimer compounds may represent the future of breast cancer therapy.

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CHAPTER I

INTRODUCION

Breast cancer is the second most commonly diagnosed malignancy among women. In 2008, more than 180,000 new cases were identified in the United States and an estimated 41,000 women died of the disease (1). Approximately 70% of primary breast cancers express estrogen receptor alpha (ER α) (2). In such cases, anti-estrogen therapy is a commonly used treatment approach. Although this therapy has proven effective in initial stages of treatment, tumors begin to show resistance due to ER α unresponsiveness after prolonged exposure to the drugs. Therefore, in order to successfully utilize anti-estrogen therapy, it is critical to know whether ER α is still active in the tumor as treatment progresses.

Furthermore, gene therapy in which genetic material is introduced in cells for therapeutic purposes is being explored as a potential method of cancer treatment (3). One specific area of study focuses on the use of non-viral, dendrimer- based vector systems to deliver DNA to various cell lines. Although the DNA-dendrimer complex has reduced transfection efficiency as compared to viral vectors, the potential for genetic recombination, toxicity, and immunogenicity is lessened with its use (4).

This thesis follows the style of Cancer Research.

Additionally, it has been shown that dendrimers have the capacity to be derivatized with a range of drugs, including those used for chemotherapy, in which form they act as drug transporters (5). Camptothecin is a chemotherapeutic agent often used in the treatment of late stage breast cancer and has shown the ability to link to a dendrimer, forming a drug-dendrimer complex. It has also been demonstrated that the dendrimer will then deliver camptothecin to the targeted tumor cells, at which point it is released (6).

The future goal of research in this area called “theranostics”, focuses on the ability of the dendrimer-based vector system to diagnose ER α responsiveness in breast cancer cells while simultaneously delivering a chemotherapeutic drug. The purpose of such a combination is twofold. First, the delivery of the drug serves to destroy cancer cells. In the process, the DNA in the dendrimer-based vector system can provide information as to whether the cells express ER α . With this information, it can be determined if anti-estrogen therapy is an effective method of treatment.

This study begins to explore the potential use of dendrimers in the treatment of breast cancer by testing the ability of the experimental compounds to deliver fluorescent reporter DNA constructs to the cancer cells.

CHAPTER II

MATERIALS AND METHODS

Cells

MCF 7 cells purchased from ATCC were used in this study. MCF 7 cells are human breast carcinoma cells that were isolated in 1970 from a 69 year old woman (7). The cells were maintained in phenol red free DMEM containing 10% FBS at 37°C in a 5% CO₂ atmosphere.

GFP plasmid

Isolated GFP plasmid was added to *E. coli* DH5alpha. The combination was placed on ice for 30 minutes, then placed at 42°C for 45 seconds, and then placed back on ice for 2 minutes. After this process, the competent bacteria had taken up the GFP plasmid. The plasmid was then purified using the Quiagen Plasmid Plus Midi Kit.

The purification of the plasmid through this process began by adding an alkaline lysate to the pelleted bacteria. The lysate was then cleared through centrifugation. After washing, the DNA was collected in the elute and precipitated with isopropanol. The result of this process is ultrapure GFP plasmid DNA.

General methods for transfection

MCF 7 cells were plated on a 96-well plate in concentrations of 30,000 cells per well. Cells incubated at 37°C in a 5% CO₂ atmosphere for 24 hours.

Following the incubation period, all wells were treated with a form of the transfection mixture containing OptiMEM, 1.5µg of DNA and a varying dendrimer concentration. The transfection mixture was removed from the cells after a period of 4-6 hours and replaced with 100µL of media. After a period of 18 hours, media was removed. The wells were then washed twice with 50µL PBS. 100µL PBS were added and the plate was then assessed for total fluorescing units using a plate reader. Following the read, fluorescence was examined under the microscope.

Positive and negative control wells were made using the transfection reagent Lipofectamine 2000 as the positive control. Lipofectamine, OptiMEM, and DNA were combined in a 1.5mL tube, allowed to incubate at room temperature for 20 minutes, and added to the positive control wells. OptiMEM and DNA were combined, allowed to incubate at room temperature for 20 minutes, and added to the negative control wells.

Modification to general transfection methods

First generation dendrimers

In this set of experiments, cells were treated with either dried or liquid dendrimer. Treatment of wells with dried dendrimer was as follows: two dendrimers (F2-1 and G2-5) of varying concentrations (n/p 5, n/p 7.5, n/p 10) were dried in a separate plate. OptiMEM and DNA were added to each well of the dried dendrimer plate and gently mixed. The plate was allowed to gently rock at room temperature throughout a 20

minute incubation period. After this period, the mixtures were added to the corresponding wells on the 96-well plate according to the appropriate dendrimer concentrations.

Two liquid dendrimers (also F2-1 and G2-5) of varying concentrations (n/p 5, n/p 7.5, n/p 10) were combined with OptiMEM and the DNA and allowed to incubate at room temperature for 20 minutes. After the incubation period, the mixtures were added to the corresponding wells according to the appropriate dendrimer concentrations.

Next generation dendrimers

In this set of experiments, OptiMEM, 1.5 μ g DNA, and varying concentrations (n/p 5, n/p 7.5, n/p 10) of one of five liquid dendrimers (M6-24, M8-24, F4-1, F4-2, F2-1) were combined and allowed to incubate for 20 minutes. After this period, the mixtures were added to the appropriate wells according to dendrimer type and concentration. The plates were incubated for 4-6 hours before the transfection mixture was removed. Following another 18 hour incubation period, the plates were analyzed via the plate reader and the microscope.

Dendrimer cytotoxicity

In a series of cytotoxicity experiments, 10,000 cells per well were plated on a 96-well plate and allowed to incubate for a period of 24 hours. Following this period, cells were treated with varying concentrations of the second generation dendrimers.

In the experiment, the negative control wells were treated with a media and water combination. Blank wells were treated with media only. The all dead control wells were treated with 30 μ L of TritonX-100.

Following treatment, the cells incubated at 37°C for 72 hours. After this period, 15 μ L of Promega G4000 MTT Dye assay were added to the cells and allowed to incubate for 4 more hours. 100 μ L of a stop solution were then added and the cells incubated for 1 more hour. Following this incubation, the cells were read with a plate reader at the absorbance of 570nm with a reference wavelength of 650nm.

CHAPTER III

RESULTS

First generation dendrimer transfection

Figure 1 represents a series of preliminary experiments illustrating the transfection capabilities of the first generation of dendrimers used (n/p 7.5). Lipofectamine showed optimal transfection as a positive control, producing 50% higher fluorescence than the negative control. When viewed under the microscope, the positive control was characterized by intense, green fluorescence.

The liquid dendrimers expressed higher levels of fluorescence than the dried dendrimers. Liquid dendrimer F2-1 showed 73% higher fluorescence than the negative control while liquid dendrimer G2-5 showed 66% higher fluorescence than the negative control. Both liquid dendrimers F2-1 and G2-5 expressed higher fluorescence than the positive control as well. However, when examined under the microscope, low-level fluorescence at an intensity much less than the positive control was observed.

Dried dendrimer F2-1 showed fluorescence 5% greater than that of the negative control while dried dendrimer G2-5 produced fluorescence 3% greater than that of the negative control. Under the microscope, little to no fluorescence was visible.

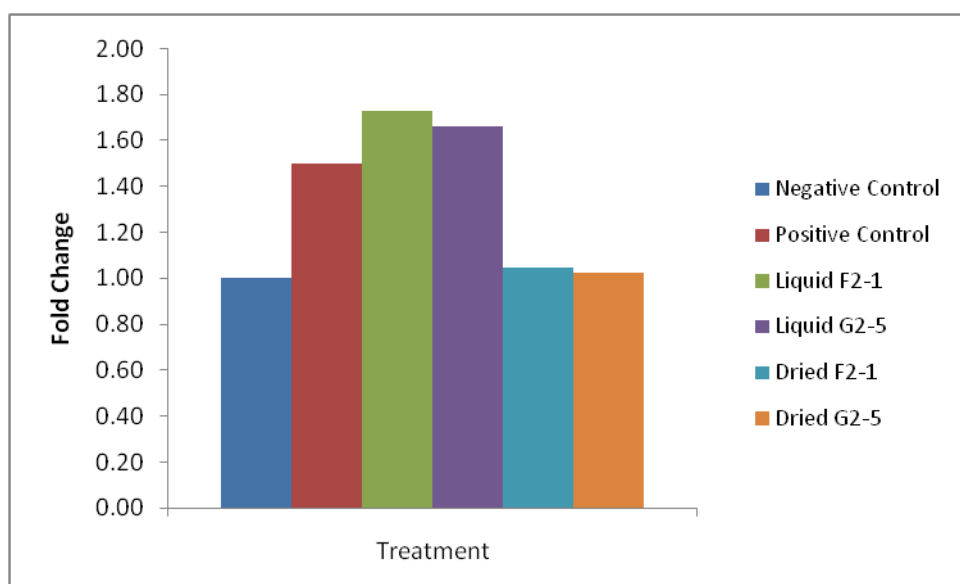


Figure 1: First generation dendrimer transfection- n/p 7.5. 30,000 cells / well were plated. Lipofectamine was used as the positive control. Following a 24 hour incubation period, cells were treated with a transfection mixture consisting of OptiMEM, 1.5 μ g GFP, and varying concentrations of dried or liquid dendrimers. After a 4-6 hour incubation period, the transfection mixture was removed and replaced with media. Plates were read for total fluorescing units after another 18 hour incubation period. Fig. 1 represents the transfection efficiency of the positive control and the dendrimers as compared to the negative control.

Second generation dendrimer transfection

Figures 2 represents a comparison of the transfection capabilities of the next generation of liquid dendrimers, M6-24, M8-96, F4-1, and F4-2 as well as dendrimer

F2-1 from the first generation at three different concentrations: n/p 5, n/p 7.5, and n/p 10.

Lipofectamine functioned as an optimal positive control, exceeding the fluorescence of the negative control by 50%. Lipofectamine again showed an intense, green fluorescence under the microscope while each of the dendrimers produced a very low-level fluorescence under the microscope.

Dendrimer concentration n/p 5

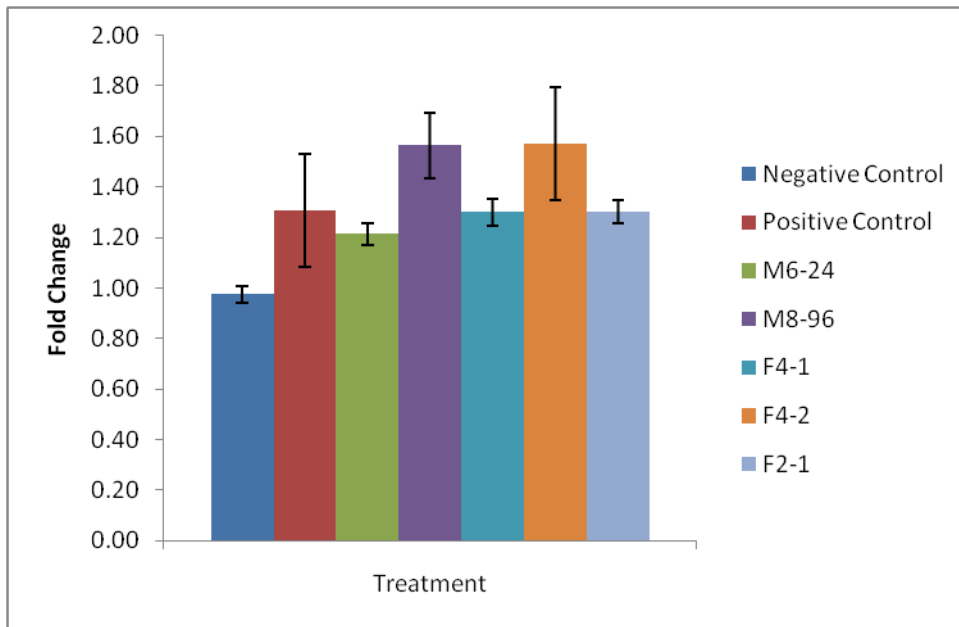
Liquid dendrimers M8-96 and F4-2 showed the highest transfection efficiencies with fluorescence at 56% and 57% higher than the negative control respectively. Each of these liquid dendrimers exceeded the fluorescence of the positive control as well. (Figure 2, Part A)

Dendrimer concentration n/p 7.5

Liquid dendrimer F4-2 again showed the highest transfection efficiency. F4-2 fluorescence was 74% higher than the negative control and exceeded the fluorescence of the positive control. (Figure 2, Part B)

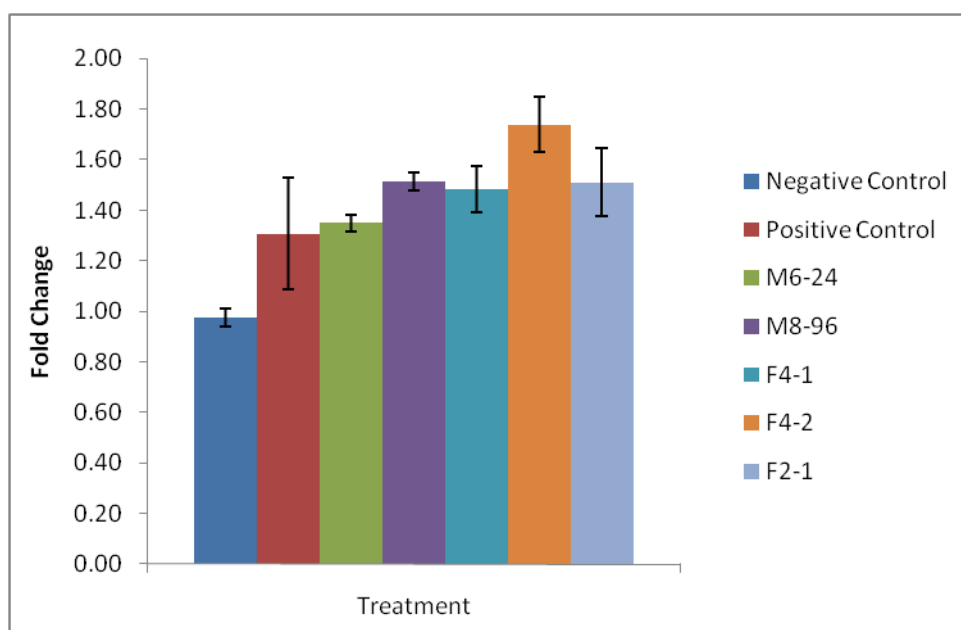
Dendrimer concentration n/p 10

Liquid dendrimers M8-96, F4-1, and F4-2 showed the highest transfection efficiencies showing 121%, 104%, and 114% higher fluorescence than the negative control respectively. All three of these dendrimers also showed more fluorescence than the positive control. (Figure 2, Part C)

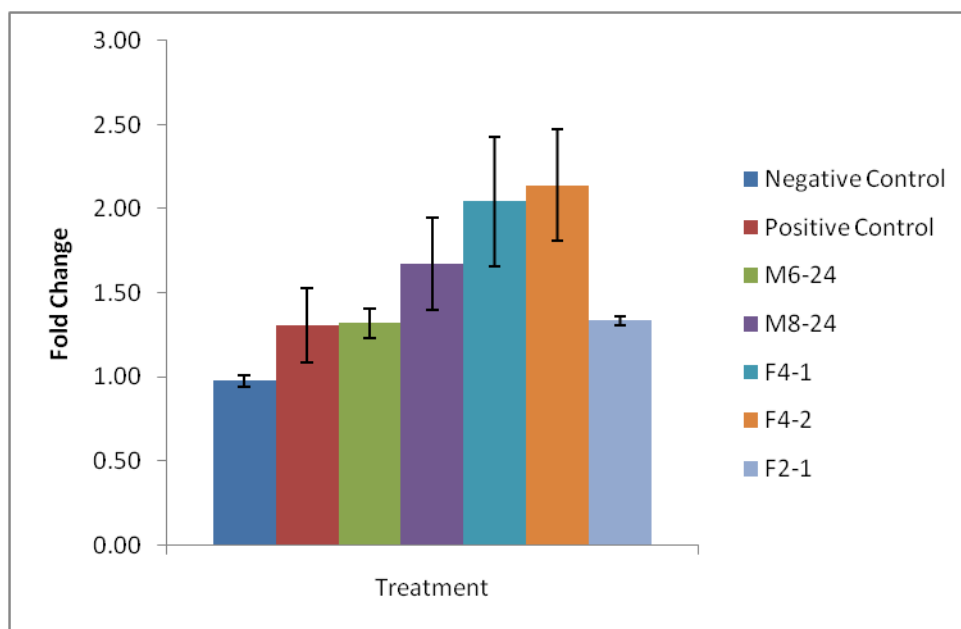


A: Second generation dendrimer transfection- n/p 5.

Figure 2: Second generation dendrimer transfection. 30,000 cells / well were plated. Lipofectamine was used as the positive control. Following a 24 hour incubation period, cells were treated with a transfection mixture consisting of OptiMEM, 1.5 μ g GFP, and liquid dendrimers at concentrations of either n/p 5, n/p 7.5, and n/p 10. After a 4-6 hour incubation period, the transfection mixture was removed and replaced with media. Plates were read for total fluorescing units after another 18 hour incubation period. Fig. 2 represents the transfection efficiency of the positive control and the dendrimers as compared to the negative control.



B: Second generation dendrimer transfection- n/p 7.5.

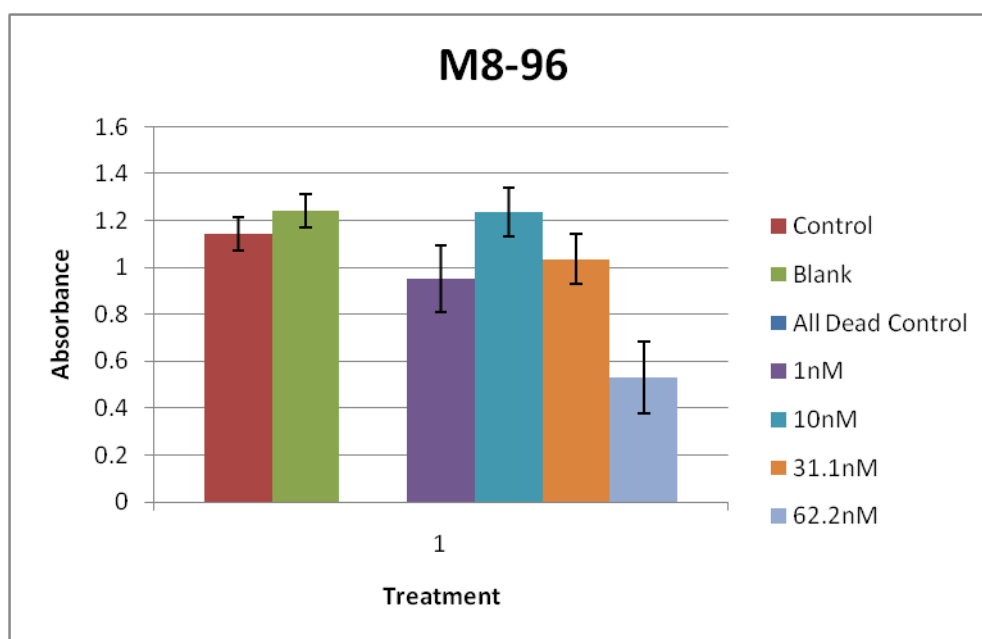


C: Second generation dendrimer transfection- n/p 10.

Figure 2 continued

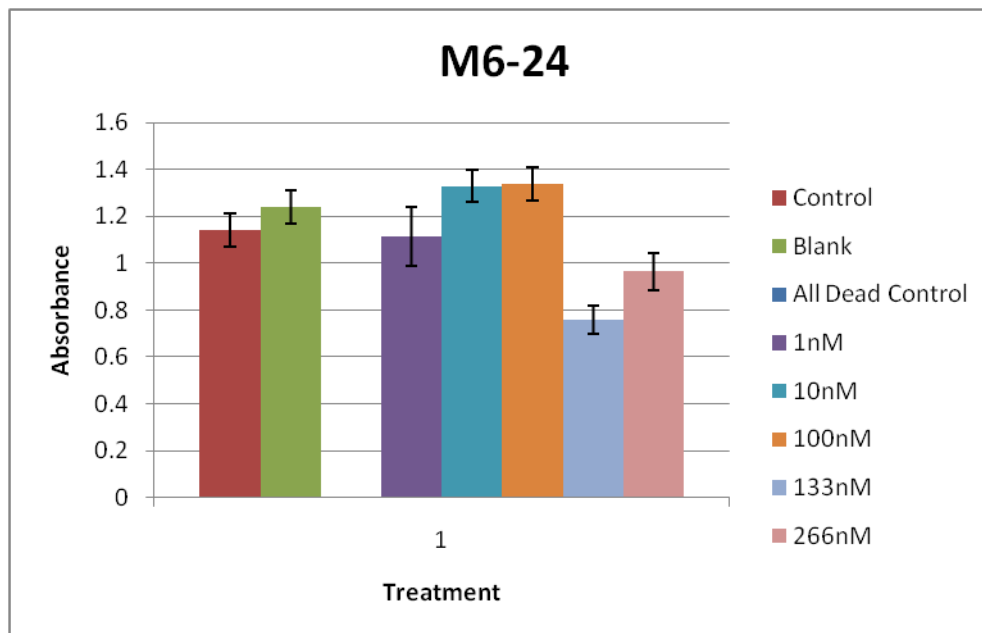
Second generation dendrimer cytotoxicity

Liquid dendrimers M8-96, M6-24, and F4-2 showed cytotoxicity at high concentrations. Specifically, dendrimer M8-96 was cytotoxic at concentration 62.2nM, M6-24 at 133nM and 266nM, and F4-2 at 134nM. The remaining dendrimers, F4-1 and F2-1, were not cytotoxic at any of the tested concentrations. (Figure 3)

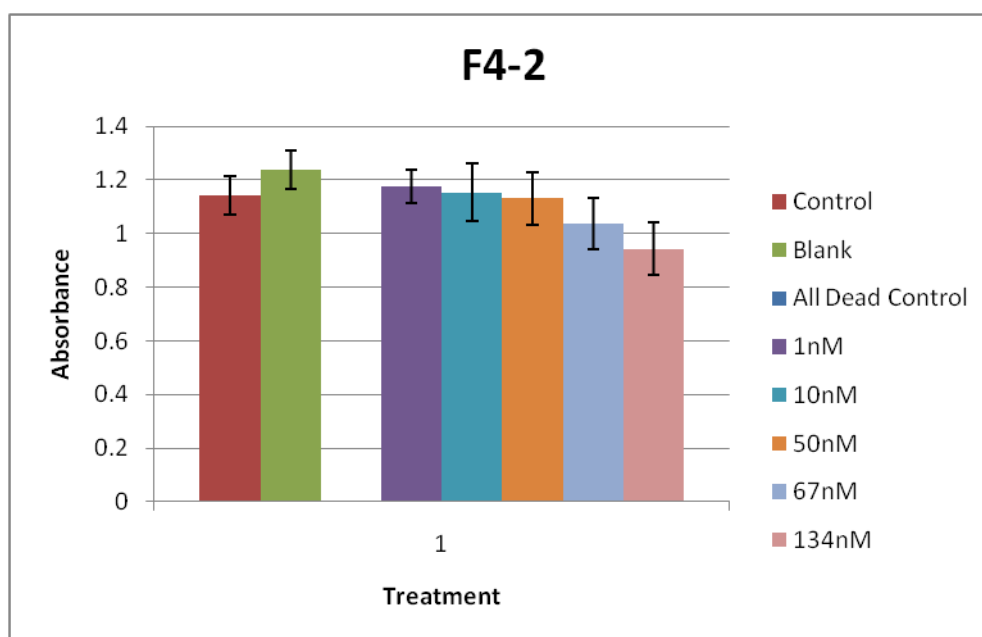


A: Second generation dendrimer cytotoxicity- M8-96

Figure 3: Second generation dendrimer cytotoxicity. MCF-7 cells were grown at 37°C. 10,000 cells / well were plated on a 96-well plate and incubated for 24 hours. Following this period, cells were treated with varying concentrations of the second generation dendrimers. Negative control wells were treated with a media and water combination. Blank wells were treated with media only. The all dead positive control wells were treated with 30µL of TritonX-100. Following treatment, the cells incubated at 37°C for 72 hours. T15µL of Promega G4000 MTT Dye assay then were added to the cells and allowed to incubate for 4 hours. 100µL of a stop solution were then added and the cells incubated for 1 hour. Cells were read with a plate reader at the absorbance of 570nm with a reference wavelength of 650nm. Data are represented according to absorbance. Lower absorbance indicates greater cell death.



B: Second generation dendrimer cytotoxicity- M6-24



C: Second generation dendrimer cytotoxicity- F4-2.

Figure 3 continued

CHAPTER IV

CONCLUSIONS

An emerging area of breast cancer research focuses on using non-viral, dendrimer-based vector systems to deliver DNA and chemotherapeutic drugs to cancer cells. The purpose of such a system would be to provide drug therapy to destroy cancer cells while also gaining knowledge of the responsiveness of ER α within the remaining cancer cells using a reporter DNA.

The purpose of this study was two-fold. The first goal was to evaluate the ability of two generations of dendrimers to deliver DNA through transfection experiments. The next goal was to determine the cytotoxic abilities of the same dendrimers.

First generation dendrimer transfection

In the initial set of experiments, dried and liquid dendrimers were used. Using dried dendrimer in a transfection experiment is a relatively new idea and this was the first experiment to gauge its transfection efficiency. Given the data obtained in this study, dendrimer transfection of MCF-7 cells appears to be more effective when liquid dendrimer is used rather than dried dendrimer. For this reason, liquid dendrimer was solely used for the remaining experiments of the study.

Second generation dendrimer transfection

In the following set of experiments, the transfection efficiency of a new generation of dendrimers was observed. Transfection of the second generation dendrimers revealed that liquid dendrimer F4-2 has the highest transfection efficiency at all concentrations tested. Additionally, liquid dendrimers M8-96 and F4-1 showed strong transfection efficiencies at higher dendrimer concentrations.

Second generation dendrimer cytotoxicity

A previous study from our laboratory showed the cytotoxic capability of dendrimer G2-NH₂ when used to treat MCF-7 cells (5). Therefore, it was of interest to determine the cytotoxic capabilities of this generation of dendrimers as well. This set of experiments revealed that liquid dendrimers M8-96, M6-24, and F4-2 are cytotoxic to MCF-7 cells at high concentrations while dendrimers F4-1 and F2-1 are not cytotoxic at the concentrations tested. Consequently, dendrimers M8-96, M6-24, and F4-2 are cytotoxic even without being derivatized to a chemotherapeutic drug.

Summary

Dendrimer- DNA- drug constructs may represent the future of breast cancer therapy. This study investigated the ability of the next generations of dendrimers to deliver reporter DNA to MCF-7 cells as well as the cytotoxic capabilities of the dendrimers. Future studies should focus on the relationship between cytotoxic dendrimers and chemotherapeutic drug transport.

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